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The inheritance of one defective BRCA1 or BRCA2 allele predisposes an individual to developing breast, ovarian and T-cell cancers. In addition, in breast cancers where BRCA1 is not mutated, it is often functionally inactivated. Furthermore, cyclin D1 has been shown to be overexpressed in many cancers including breast cancer and its associates with BRCA1. Because of the crucial role of both of these proteins in cancer, it is reasonable to expect that this interaction has a significant role in tumor cells. The understanding of when this interaction occurs during cell cycle progression will help to determine the role of cyclin D1/BRCA1 binding in breast cancer cells. Therefore, I hypothesize that the direct interaction of cyclin D1 with BRCA1 results in the cell cycle dependent regulation of the activity of BRCA1. In this study, I wish to identify and confirm the cell cycle dependent cyclin D1/BRCA1 interaction in breast cancer cells, determine the biochemical consequence of cyclin D1/BRCA1 interaction in breast cancer cells, and determine the functional consequence of BRCA1 phosphorylation in breast cancer. BRCA1's phosphorylation by cyclin D1/cdk complexes may help to regulate BRCA1's localization to the nucleus, since BRCA1 has been shown to have a cytoplasmic expression pattern, but acts primarily in the nucleus. Phosphorylation may also be important in modulating BRCA1's ability to bind DNA, either as a transcription factor or as part of a DNA damage repair complex. Determining the consequences of the interaction of cyclin D1/BRCA1 could lead to a more complete understanding of how breast cancer occurs, thus leading to new treatment options.

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INTRODUCTION

Cyclins and their catalytic partners, cyclin dependent kinases (cdks), are some of the major players in cell cycle control. Cyclin/cdk complexes are positive regulators of the cell cycle, helping the cell to progress through the G_1/S and G_2/M checkpoints. For instance, progression through the G_1/S checkpoint is accomplished mainly through the phosphorylation of the retinoblastoma protein (Rb). Rb is a tumor suppressor protein that acts by binding and thus sequestering proteins such as E2F, c-Abl, and HDAC1 (1-3). Upon Rb phosphorylation, proteins such as E2F bind to DNA specific promoter sites and initiate transcription of cell proliferation genes (4). Cyclin D/cdk4,6, cyclin E/cdk2, and cyclin A/cdk2 complexes are all activated at different times in the cell cycle and have the ability to differentially phosphorylate Rb (5-7). This differential phosphorylation of Rb allows for the release of bound Rb proteins, thus regulating the transcription of specific genes such as cyclin E and cyclin A (8).

The cyclin dependent kinase inhibitors (CKIs) negatively regulate progression through the cell cycle. CKIs bind to cyclin/cdk complexes, inhibit their kinase activity and cause the cell cycle to slow down and even to be blocked at cell cycle checkpoints. Blocking the cells at different cell cycle checkpoints allows the cell to fix any mutations or DNA damage that may have occurred before it proceeds into the next stage of the cell cycle (4).

Cell cycle regulatory genes are often targeted in tumorigenesis mainly because this is one way of deregulating and increasing cell proliferation (9, 10). Many cyclins and CKIs have been implicated in cancer (11, 12). The cyclin Ds in particular have been implicated in a variety of cancers such as breast and prostate cancers, and T-cell leukemia (13-15). The chromosome band 11q13, which contains the cyclin D1 gene, is amplified in 15-20% of breast cancer cases (16). In addition, the protein levels of cyclin D1 have been shown to be overexpressed in up to 35% of breast cancers (17). Finally, cyclin D1 has been shown to act as an oncogene through a study where the expression of cyclin D1 in mouse mammary glands led to tumor formation (18).

In addition to cyclin D1, BRCA1 is a major player in the progression to breast cancer. BRCA1 and BRCA2 both encode a large nuclear protein (1863 and 3418 amino acids, respectively). These tumor suppressor proteins are expressed in many tissues and are most abundant during S/G_2 phase of the cell cycle. The inheritance of one defective BRCA1 or BRCA2 allele predisposes an individual to developing breast, ovarian, and T-cell cancers (19). Hereditary cancer constitutes only about 5-10% of all breast cancer cases and only 80% of these cases have mutations in either BRCA1 or BRCA2 (20, 21). Although the remaining 90-95% of cases, considered sporadic breast cancer, rarely have BRCA1 mutations, the BRCA1 protein can be functionally inactivated in these cases (22).

Homozygosity for targeted mutations in murine BRCA1 or BRCA2 has been shown to precipitate defective cell division, chromosomal instability, and hypersensitivity to genotoxins, which is indicative of defects in DNA repair (23-26). BRCA1 interacts with a multitude of proteins including tumor suppressors, oncogenes cell cycle regulators and transcriptional activators (27). Most notable is the interaction of BRCA1 with proteins involved in DNA repair such as RAD50 and ATM (28, 29). Therefore, BRCA1 has been characterized as a caretaker of the genome.

Based on the role of BRCA1 as a tumor suppressor and in DNA damage control, it has been suggested that BRCA1 regulates cell proliferation through regulation of cell cycle checkpoints (30). BRCA1 has been shown to be phosphorylated in a cell cycle dependent manner, with its hyperphosphorylated form dominating in the late G_1 and S phases (31, 32).

BRCA1 interacts with Rb and can be phosphorylated by cyclin A/cdk2 complexes, which aids in S phase progression. MacLachlan *et al.* (33) have shown that ectopic expression of BRCA1 leads to dephosphorylation of Rb, while Wang *et al* (34) have shown that Rb regulates the BRCA1 gene through its modulation of E2F transcriptional activity. Even though various cyclin/cdk complexes have been shown to phosphorylate BRCA1, the consequence of this phosphorylation on BRCA1's activity, cell cycle progression, or proliferation has not been clearly defined.

The long-term goal of this research project is to understand how protein-protein interactions affect the function of the BRCA1 protein. Understanding which proteins aid in the tumor suppressor function of BRCA1 will help to identify which pathways may be altered in a developing mammary tumor. In particular, cyclin D1 has been shown to physically interact with BRCA1, which suggests that BRCA1 may have a critical role in cell cycle progression (31, 35). This proposal will determine the functional consequences of cyclin D1/BRCA1 interaction. My hypothesis is that the direct interaction of cyclin D1 with BRCA1 results in the cell cycle dependent regulation of the activity of BRCA1. BRCA1's phosphorylation by cyclin D1/cdk complexes may help to regulate BRCA1's localization to the nucleus, since BRCA1 has been shown to have a cytoplasmic expression pattern, but acts primarily in the nucleus. Phosphorylation may also be important in modulating BRCA1's ability to bind DNA, either as a transcription factor or as part of a DNA damage repair complex. Determining the consequences of the interaction of cyclin D1/BRCA1 could lead to a more complete understanding of how breast cancer occurs, thus leading to new treatment options.

In my previous annual report, I standardized various techniques such as immunoprecipitations, kinase assays, and confocal microscopy that are vital to the completion of my aims. I also initiated experiments to analyze the proteins levels of cyclin D1 and BRCA1 in two different breast cancer cell lines as well as to detect the interaction between these two proteins in both cell lines. In this report, I show progress from March 2004-March 2005. I will show that the regions of BRCA1 that are important for cyclin D1 binding and phosphorylation. In addition, I will present data that indicates that the interaction between cyclin D1 and BRCA1 occurs throughout the cell cycle. My studies also demonstrate that cyclin D1/cdk4 complexes phosphorylate BRCA1 *in vivo*. Finally, I will provided evidence that cyclin D1 and BRCA1 co-localize *in vivo*.

BODY

Experimental Methods

Cell culture. MCF-7, and T47D are breast cancer epithelial cells derived from pleural effusions (36). MCF-10A is a normal breast epithelial cell line. T47D cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% streptomycin/penicillin. MCF-7 cells were grown in DMEM containing 10% (FBS), 1% L-glutamine, and 1% streptomycin/penicillin. MCF-10A cells were grown in Complete Mammary Epithelial Cell Medium plus 100 ng/ml of cholera toxin. All cells were grown in a 5% CO_2 incubator at 37°C. G_0 synchronization was performed as follows. Briefly, cells were brought to 95 % confluency, washed, and cultured in serum-free DMEM for 3 days. To stimulate cells DMEM plus 20% heat inactivated fetal calf serum (HIFCS) was added. MCF-7 cells were synchronized in S phase by treating with Hydroxyurea (2mM) for 48 hours at 60% confluency. Cells were released into DMEM plus 20% HIFCS and collected 4 hours later. G2/M phase cells were obtained by treating MCF-7 cells with 50 ng/ml of Nocodazole for 24 hours and collected the cells at 0hr.

Plasmids. pDC78 GST-BRCA1 (1-500), pDC79 GST-BRCA1 (452-1079), pDC80 GST-BRCA1 (1021-1552), pDC81 GST-BRCA1 (1501-1861), pDC99 GST-BRCA1 (504-802), pDC208 GST-BRCA1 (697-1276) were a kind gift from Dr. Tanya Paull at the University of Texas/ICMB. These constructs were original made in Dr. Elledge's lab at Vanderbilt University (37).

Transformation of GST-Plasmids. One μg of specified plasmid was incubated on ice for 2 hours with 200μl of the DH5α strain of *E. coli* competent cells. Cells were then heat shocked at 42°C for 90 seconds. Three hundred μl of LB broth (-Amp, +$MgSO^4$, +dextrose) was added to the solution and the cells were incubated for 30 minutes at 37°C. Two hundred μl of solution was spread on Amp LB agar plates for selection. Plates were incubated overnight at 37°C. The next day, a single colony was selected and placed in 10ml LB broth (+Amp, +$MgSO^4$, +dextrose), and shaken overnight at 37°C. Then, 5ml of *E. coli* was transferred into 150-200ml of LB broth (+Amp, +$MgSO^4$, +dextrose) and shaken until the solution reached an OD of 0.5 to 0.6. The cells were then induced with 0.5 mM IPTG at 25°C overnight. After induction, *E.coli* was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C.

Purification of GST-Proteins. *E. coli* pellets were resuspended in 10 ml of PBS + 1% Triton X 100. *E.coli* was then lysed through sonication and freeze/thaw methods. After last thaw, the solutions were centrifuged at 10,000 rpm for 10 minutes. Supernatant was transferred to new tubes and pellet was discarded. Glutathione-Sepharose Beads were prepared by washing 3 times with PBS+1% Triton X-100, and a 30% slurry was created. Then, 100-200μl of a Glutathione-Sepharose Bead solution was added to 2-3 ml of lysed E. coli supernatant for binding overnight at 4°C. The next day, complexes were washed once in TNE300 + 0.1% NP-40 and once in PBS+1% Triton X-100. Complexes were resuspended in PBS + 1% Triton X-100. An aliquot of each GST-fusion protein was run on a 4-20% Tris-glycine gel by SDS-PAGE along with standard protein controls (BSA). Coomassie Blue staining was used to determine relative amounts of GST-protein as compared to standard protein concentrations.

TNT Binding Assays. ^{35}S-labeled HA-cyclin D1 was produced using the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's protocol. pcDNA3-HA cyclin D1 [a kind gift from Dr. Doris Germain (38)] was used to produce the ^{35}S-labeled protein. To confirm synthesis, proteins were separated by SDS-PAGE on a 4-20 % Tris-glycine polyacrylamide gel. The gels were dried and exposed to a PhosphorImager cassette. Binding assays included GST-BRCA1 constructs (1 μg), or GST (5 μg) (concentration determined by Coomassie Blue staining) and 5 μl of TNT reactions and were carried out at 4°C overnight. The next day, complexes were washed twice with TNE_{150} + 1.0% NP-40 and once with TNE_{50} + 0.1% NP-40. Complexes were separated on a 4-20% Tris-glycine gel by SDS-PAGE. The gel was dried and exposed to a PhosphorImager cassette.

Cell extract preparation. Cells were centrifuged at 4°C for 5 minutes at 2,000 rpm. Cell pellets were washed twice with D-PBS without Ca2+ and Mg2+. Cell pellets were then resuspended in lysis buffer (50 mM Tris-Cl, pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (NP-40), 50 mM NaF, 1 mM DTT, 0.2 mM Na_3VO_4 and one complete tablet of protease cocktail inhibitor/ 50 ml buffer) on ice and vortexed every 5 minutes for a total of 30 minutes. Cell lysates were then transferred to Eppendorf tubes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was then transferred to a new Eppendorf tube and the protein concentration taken using Bio-Rad protein assay.

Antibodies and Immunoblotting. Anti-cyclin D1 (M-20) and anti-BRCA1 (C-20) polyclonal antibodies were purchased from Santa Cruz. Total cellular protein was separated on 4-20% Tris-glycine polyacrylamide gels and transferred to a polvinylidene difluoride (PVDF) membranes (Immobilon-P transfer membranes; Millipore Corp.) overnight at 0.08 A. For the last thirty minutes of transfer, the amperage was increased to 200 A. Following the transfer, blots were blocked with 5% non-fat dry milk in 50 ml of TNE_{50} (100 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) plus 0.1% NP-40. Membranes were probed with a 1:200 dilution of antibodies at 4°C overnight, followed by three washes with TNE_{50} plus 0.1% NP-40. Next day blots were incubated with 10 ml of ^{125}I-protein G (Amersham, 50 μl/10ml solution) in TNE_{50} plus 0.1% NP-40 for 2 hrs at 4°C. Finally, blots were washed three times in TNE_{50} plus 0.1% NP-40 and placed on a PhosphorImager Cassette for further analysis.

Immunoprecipitation. One mg of cellular proteins and 5μg of appropriate antibody were used. Samples were rotated overnight at 4°C and the next day protein A and protein G agarose beads (Oncogene Research Products/Calbiochem catalog IP05) were added. This mixture was rotated for 90 minutes at 4°C. Samples were washed twice in the appropriate TNE buffer plus 0.1% NP-40 and once in TNE_{50} plus 0.1% NP-40. Complexes were analyzed by reducing SDS-PAGE, on a 4-20% Tris-glycine gel.

Kinase assays. Cell extracts were immunoprecipitated (IP) overnight with the anti-cyclin D1 (M-20) rabbit polyclonal antibody. Protein G and protein A agarose beads were added to IPs and rotated for 2 hrs at 4°C. IPs were washed twice with the TNE_{300} buffer, once with TNE_{50} + 0.1% NP-40, and twice with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 50 mM NaF, 0.2 mM Na_3VO_4 and one complete tablet of protease cocktail inhibitor/ 50 ml buffer). The appropriate substrate was added (GST-Rb or various GST-BRCA1 fusion proteins)

to each tube (1μg), with 1 μl of [γ^{32} P]-ATP (3000 Ci/mmol). Reactions were incubated at 30°C for 1 hour and stopped by the addition of 15 μl of 2X SDS sample buffer. The samples were separated by reducing SDS-PAGE on a 4-20% Tris-glycine gel. Gels were stained with Coomassie blue, destained, and then dried for 2 hr. Following drying, they were exposed to a PhosphorImager cassette and analyzed utilizing Molecular Dynamic's ImageQuant Software.

Chromatin Immunoprecipitation Analysis (ChIP). Chromatin immunoprecipitations were performed using a modification of previously published methods (36,37). Approximately 5×10^6 cells were utilized per immunoprecipitation. Cells were cross-linked by addition of formaldehyde (1% final concentration). Cross-linking was allowed to proceed at 37° C for 10 min. Cells were washed with PBS three times. Next, cells were collected by trypsin and scraping. Cells were collected by centrifugation at 4000 rpm for 10 min, and the resulting pellet was lysis in 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 7.5) and incubated 10 min at room temperature. Lysates were sonicated on ice for 10 cycles to obtain an average DNA length of 500 to 1200 bp. Lystates were then cleared by centrifugation at 4000 rpm for 10 min and diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 7.5, 167 mM NaCl). Chromatin was pre-cleared with a mixture of protein A and protein G Sepharose (blocked previously with 1 mg/ml salmon sperm DNA and 1 mg/ml BSA) at 4°C for an hour. Pre-cleared chromatin was incubated with 5 μg of antibody at 4°C overnight. Next day, 60 μl of a 30% slurry of blocked protein A/G sepharose was added, and immune complexes were recovered. Immunoprecipitates were washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL, pH 7.5, 150 mM NaCl) twice with High salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL, pH 7.5, 600 mM NaCl), once with LiCl wash buffer (0.25 M LiCl, 1 mM EDTA, 1% NP-40, 1.0% deoxycholate, 10 mM Tris-HCl, pH 7.5), and once with TE buffer. IPs were eluted twice with elution buffer (1.0% SDS, 0.1 M NahCO3) at room temperature for 15 minutes each. To the eluants and input, 20 μl of 5 M NaCl was added and cross-links were reversed by incubating samples at 65°C overnight. Next day, proteinase K (100 ug/ml) was added and samples incubated at 55°C for one hour. Samples were extracted with phenol:chloroform and ethanol precipitated. Pellets were resuspended in 50 μl of TE buffer and assayed by semi-quantitative PCR. Thirty-five cycles of PCR were performed in 50 μl with 10 μl of immunoprecipitated material, 10 pmole of primers and 1 units of *Taq* DNA polymerase. Finally, PCR products were electrophoresed on 1% agarose gels and visualized.

Immunofluorescent staining. MCF-7 cells were grown on coverslips and washed twice with D-PBS without Mg^{2+} and Ca^{2+}. Cells were fixed 20 minutes with 4% paraformaldehyde. Next, cells were permeabilized for 20 minutes with 0.2% Triton X-100 in D-PBS without Mg^{2+} and Ca^{2+}. Slides were then washed with D-PBS without Mg^{2+} and $Ca^{2+}$and blocked for 10 minutes with 10% bovine serum albumin (BSA). Coverslips were incubated for one hour at 37°C with the primary antibody, (1:200 dilution) in 10% BSA. Primary antibodies used were anti-cyclin D1 (M-20) rabbit polyclonal and anti-BRCA1 (Ab1) mouse monoclonal. Slides were then washed three times with D-PBS without Mg^{2+} and Ca^{2+}. Secondary antibodies (1:50), in 10% BSA, were added and the slides were again incubated for 1 hour, in the dark at 37°C. Secondary antibodies used were Fluor 488-goat anti-rabbit IgG and Texas Red (TR)-goat anti-mouse IgG from Molecular Probes. The previous washes were repeated. Slides were then incubated at room temperature for 20 minutes with 2 μM of TOTO-3, a dimeric cyanine nucleic acid stain (Molecular Probes), for nuclear staining. Slides were washed briefly with H_2O and the excess

liquid was removed. Prolong anti-fade (Molecular Probes) was added to the slides to prevent photo-bleaching. After drying the coverslips were sealed.

Confocal laser scanning microscopy. Slides were viewed with a Bio-Rad MRC1024 confocal laser scanning microscope (Center for Microscopy and Image Analysis, George Washington University) using the 60 X objective. Optical sections were taken using z-dimensions of 1.0 μm. Pictures were produced using Adobe Photoshop 5.0 and Bio-Rad plug-ins.

Results and Discussion

Aim 1. Identify and confirm the cell cycle dependent cyclin D1 and BRCA1 interaction in breast cancer cells.

Task 1. Initial experiments were designed to confirm that cyclin D1 and BRCA1 physically interact *in vivo*. Cyclin D1 has been shown to be overexpressed in many cancers, including breast cancer. Its association with BRCA1 has been demonstrated (31, 35). Because of the crucial role of both of these proteins in cancer it is reasonable to expect that this interaction has a significant role in the tumor cell. The understanding of when this interaction occurs during cell cycle progression will help to determine the role of cyclin D1/BRCA1 binding in breast cancer cells.

In order to initially address this aim, my first set of experiments examined the endogenous protein levels of BRCA1 and cyclin D1 in breast cancer cell lines. The breast cancer cell lines utilized were MCF-7 and T47D. MCF-7 and T47D are breast cancer epithelial cells derived from pleural effusions (36). As was proposed in Task 1 of Aim 1, asynchronous cultures were used to prepare protein extracts. The levels of cyclin D1 and BRCA1 were examined by immunoblotting utilizing anti-cyclin D1 and anti-BRCA1 rabbit polyclonal antibodies. Results from the western blot analysis indicate that the expression of cyclin D1 and BRCA1 is variable between cell lines. Please note that T47D cells expressed higher levels of BRCA1 compared to MCF-7 cells, whereas MCF-7 cells expressed higher levels of cyclin D1 as compared to T47D cells (Figure 1A). These results are interesting because the different expression levels of both cyclin D1 and BRCA1 endogenous proteins can be utilized to study effects of protein levels on their binding.

I next examined the ability of cyclin D1 to bind to BRCA1 in MCF-7 and T47D cells through immunoprecipitation assays followed by western blotting. Anti-BRCA1 polyclonal antibodies were utilized in the immunoprecipitation assays, with Protein A and G agarose beads alone serving as the negative control. The immune complexes were examined by western blotting for cyclin D1. My results show that cyclin D1 was observed in immune complexes obtained by anti-BRCA1 antibodies (Figure 1B). However, some cyclin D1 was detected in the beads only control. Therefore, the stringency of wash conditions was increased up to 600 mM salt and 1% NP-40. At these conditions, cyclin D1 maintained binding to BRCA1. Even though there was still some non-specific binding to the protein A and G agarose beads after this wash, the binding of cyclin D1 to BRCA1 was significantly higher than the background (Figure 1B, compare lanes 3 and 4 and lanes 5 and 6).

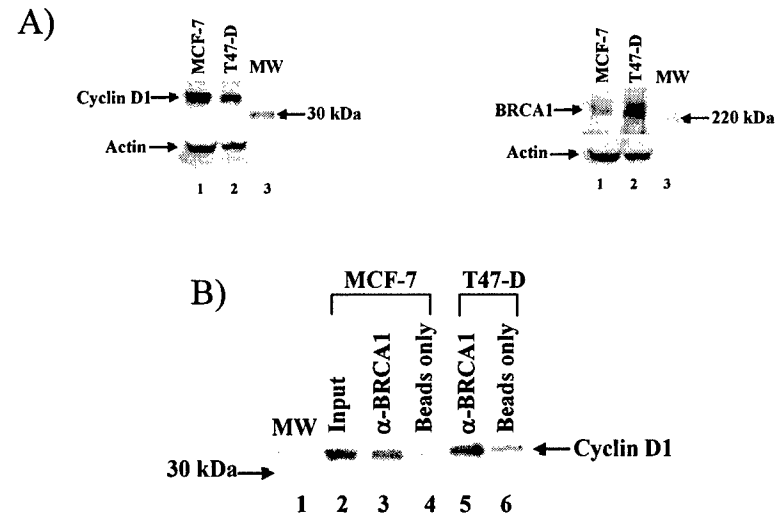


Figure 1: Cyclin D1 associates with BRCA1. A) Seventy five micrograms of total cellular protein was separated by SDS-PAGE on a 4-20% Tris-glycine gel, transferred, and blotted with anti-cyclin D1 and anti-BRCA1 rabbit polyclonal antibodies. The blot was stripped and reprobed with anti-actin rabbit polyclonal antibodies. The antigen-antibody complex was further detected with 125I-Protein G. B) MCF-7 and T47D cell extracts (1 mg) were Immunoprecipitated with anti-BRCA1 rabbit polyclonal antibody or no antibody overnight at 4°C. The complexes were precipitated with protein A+G agarose beads and washed with TNE600 + 1.0% NP-40. Proteins were then separated by reducing SDS-PAGE on a 4-20 % Tris-glycine gel and transferred onto a PVDF membrane. Western blot was performed with anti-cyclin D1 rabbit polyclonal antibody.

To confirm these interactions, I performed in vitro binding assays with Glutathione S-transferase (GST) -BRCA1 fusion proteins and ^{35}S labeled cyclin D1. Six GST-BRCA1 fragments spanning the BRCA1 protein were obtained from Dr. Tanya Paull (37). The GST-BRCA1 constructs were transformed into Escherichia coli and expressed as fusion proteins with glutathione S-transferase (GST). The fusion proteins were then purified on glutathione-Sepharose beads. For a negative control, GST alone was expressed and purified similarly. ^{35}S labeled cyclin D1 was *in vitro* transcribed and translated using Promega's TNT kit.

GST-BRCA1 proteins were incubated with ^{35}S-cyclin D1 overnight. The next day protein complexes were washed and analyzed by SDS-PAGE. Results of a typical binding experiment are shown in Figure 2A. I observed cyclin D1 binding to multiple regions of BRCA1. There was strong binding observed on fragments 1-500, 504-802, and 452-1079. It is interesting to note that BRCA1 (504-802) is encompassed within the 452-1079 region indicating that this smaller fragment is the region that is necessary for binding. Also, it appears as though there is a separate binding site in the N-terminus of BRCA1 (1-500). Little or no binding was observed with fragment 1021-1552. Average binding of cyclin D1 to BRCA1 fragments is shown in Figure 2B. These results represent the average of five independent experiments. *Collectively, these results indicated that BRCA1 and cyclin D1 interact in vivo and that regions 1-500 and 504-802 of BRCA1 are particularly important for binding in vitro.*

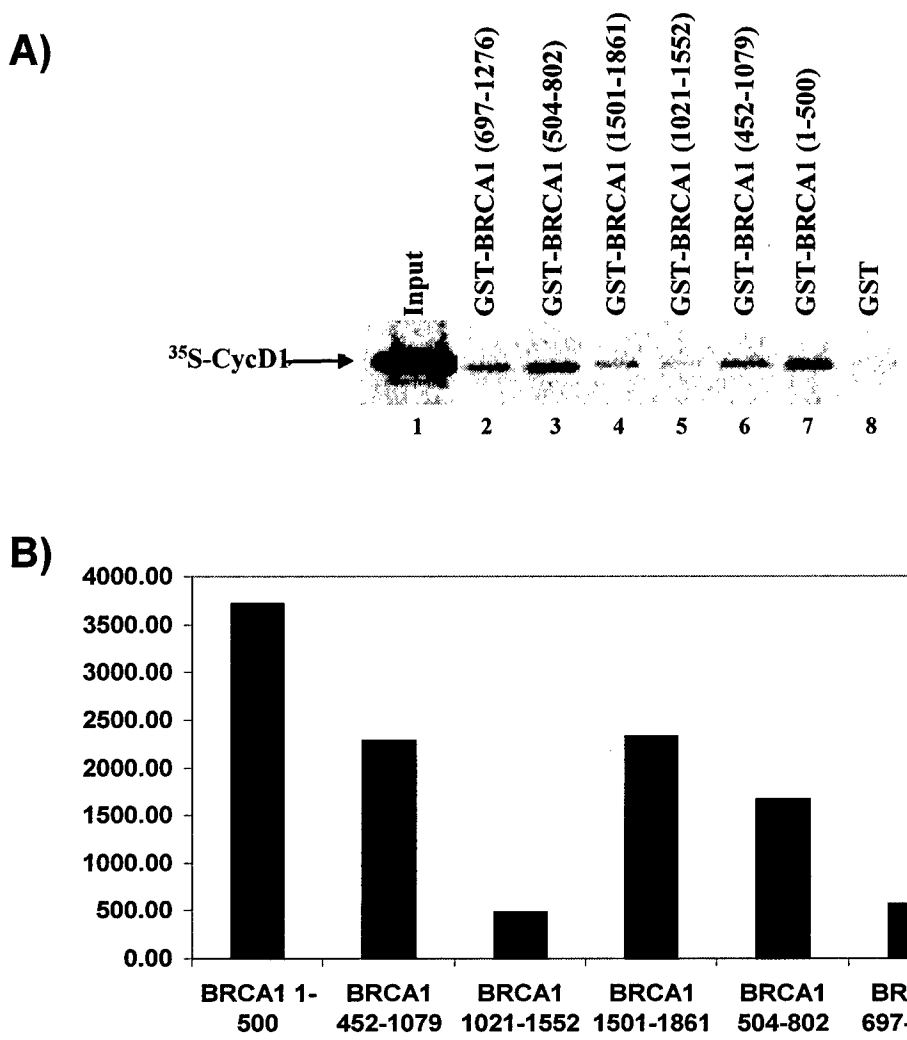


Figure 2: Cyclin D1 binds BRCA1 in vitro. A) 35S-labeled cyclin D1 (35S-CycD1) was incubated with various GST-BRCA1 constructs (1.0 ug) or GST (2 ug). All GST tagged protein amounts were normalized to allow for quantitative comparison. Binding assays were carried out with GST-proteins purified from *E. coli* and 5 µl of TNT reactions at 4°C overnight. The next day, complexes were washed (TNE150 + 0.1% NP-40), separated by SDS-PAGE on a 4-20 % Tris-glycine gel, dried, and exposed to a PhosphorImager cassette. B) Average of five in vitro binding assay. Relative binding was calculated using ImageQuant Software.

Task 2. In order to determine the cell cycle dependent interaction between cyclin D1/BRCA1, MCF-7 cells were analyzed at various points in the cell cycle. Asynchronous cells, G0/G1 cells (synchronized in G0 by serum starvation), S phase cells (treated with HU and picked up 4 hours after release), and G2/M cells (treated with Nocodazole) were collected. Cells were stained with propidium iodine and analyzed by FACS analysis to ensure proper synchronization (Figure 3A). As can be seen in Figure 3A, an asynchronous MCF-7 population consists of 55.68% G0/G1 cells, 25.88% S phase cells, and 18.44% G2/M phase cells. Upon serum starvation for 3days in 0% FBS, the G0/G1 population could be enriched to 88.62%. MCF-7 cells treated with 2mM Hydroxyurea, released into complete media and picked up 4 hours later, contained 73.63% of the cells in S phase. Finally, cells treated with Nocodazole for 24 hrs were 85.45% at G2/M phases.

Western Blotting was performed on the various cell cycle populations (Figure 3B). Both cyclin D1 and BRCA1 displayed cell cycle dependent expression, with cyclin D1 being most highly expressed at G1 phase and BRCA1 at S phase (Figure 3B, lanes 3 and 2 respectively). Next, immunoprecipitation/western blot analysis was performed on the cell cycle time point samples (Figure 3C). Unexpectedly, cyclin D1 and BRCA1 were found to interact throughout the cell cycle. It is possible that this interaction only becomes functionally important at certain stages or in response to a stress signal. It is interesting to note that similar results were also obtained with MCF-10A cells (data not shown). *In summary, I have observed that cyclin D1 and BRCA1 interact throughout all the stages of the cell cycle.*

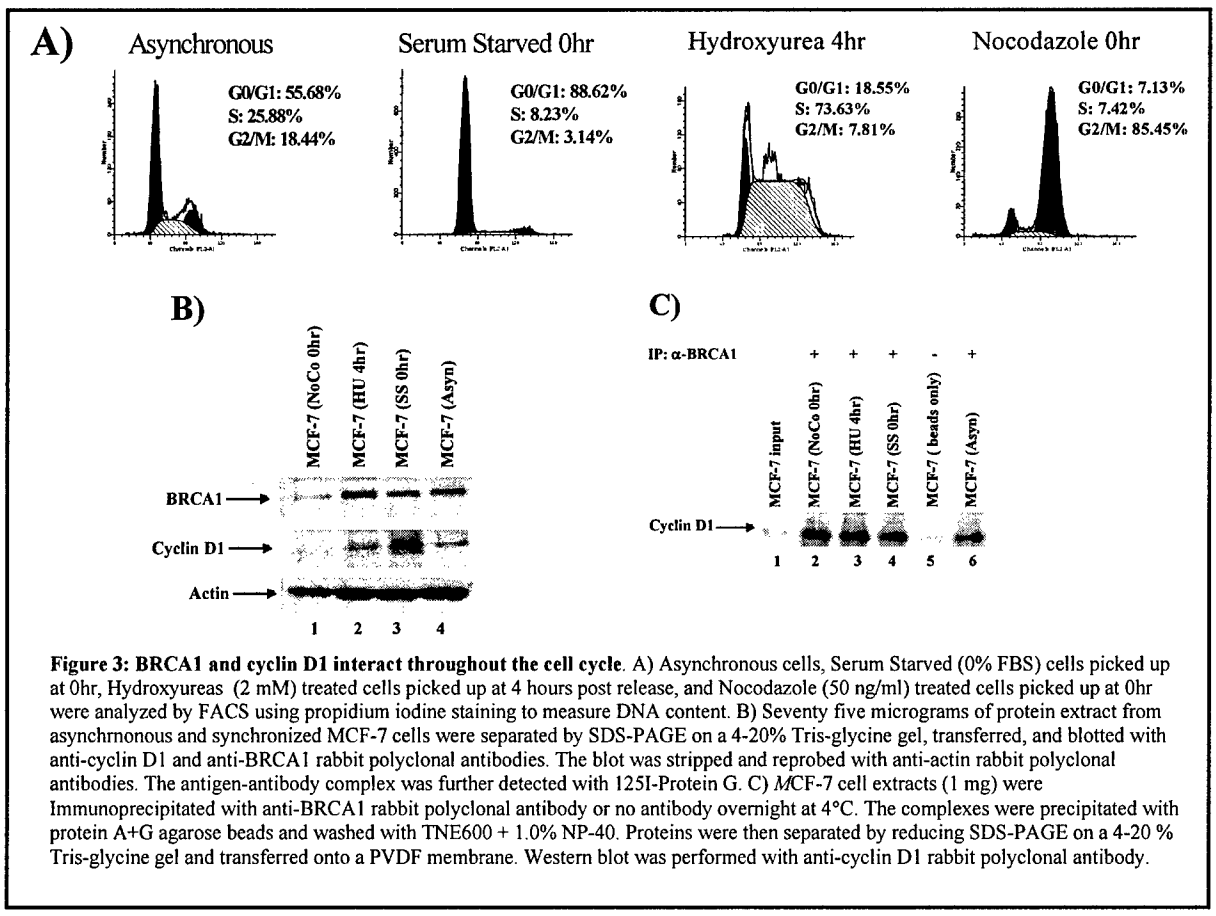
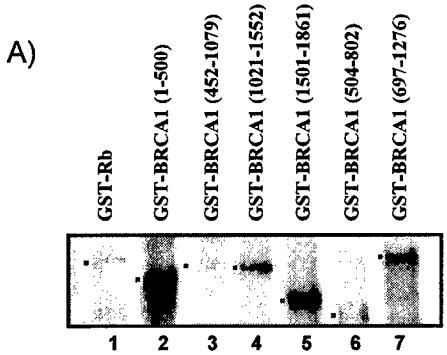


Figure 3: BRCA1 and cyclin D1 interact throughout the cell cycle. A) Asynchronous cells, Serum Starved (0% FBS) cells picked up at 0hr, Hydroxyureas (2 mM) treated cells picked up at 4 hours post release, and Nocodazole (50 ng/ml) treated cells picked up at 0hr were analyzed by FACS using propidium iodine staining to measure DNA content. B) Seventy five micrograms of protein extract from asynchronous and synchronized MCF-7 cells were separated by SDS-PAGE on a 4-20% Tris-glycine gel, transferred, and blotted with anti-cyclin D1 and anti-BRCA1 rabbit polyclonal antibodies. The blot was stripped and reprobed with anti-actin rabbit polyclonal antibodies. The antigen-antibody complex was further detected with 125I-Protein G. C) *M*CF-7 cell extracts (1 mg) were Immunoprecipitated with anti-BRCA1 rabbit polyclonal antibody or no antibody overnight at 4°C. The complexes were precipitated with protein A+G agarose beads and washed with TNE600 + 1.0% NP-40. Proteins were then separated by reducing SDS-PAGE on a 4-20 % Tris-glycine gel and transferred onto a PVDF membrane. Western blot was performed with anti-cyclin D1 rabbit polyclonal antibody.

Aim 2. Determine the biochemical consequence of cyclin D1/BRCA1 interaction in breast cancer cells.

Task 1. One possible consequence of BRCA1/cyclin D1 interaction is the phosphorylation of BRCA1. This hypothesis is based on the facts that BRCA1 is a phosphoprotein and has been shown to have multiple cdk consensus phosphorylation sites along with nine RXL motifs, which are involved in the docking of cyclin/cdk complexes. In addition, it has been previously shown that BRCA1 is phosphorylated by cyclin A/cdk2 (*in vivo)*, cyclin E/cdk2 (*in vitro*), and cyclin D1/cdk (*in vitro)* (22, 35). Therefore, I will re-confirm and provide further details about the phosphorylation of BRCA1 by cyclin D1/cdk4 complexes.

The functional activity of cyclin D/cdk complexes is often measured through *in vitro* kinase assays. Therefore the ability of cyclin D/cdk complexes to phosphorylate BRCA1 was measured utilizing *in vit*ro kinase assay as has been outlined in Task 1 of specific Aim 2. GST-Rb was used as a positive control for cyclin D1/cdk4 kinase activity. One representative kinase assay is shown in Figure 4A, whereas the average of multiple kinase assays is indicated in Figure 4B. Cyclin D1/cdk complexes were observed to phosphorylate all GST-BRCA1 constructs tested, but to varying extents. GST-BRCA1 (1-500) consistently exhibited high levels of phosphorylation, whereas GST-BRCA1 (1021-1552) and (697-1276) showed lower levels of phosphorylation. BRCA1 fragments (452-1070) and (504-802) on average became significantly phosphorylated (Figure 4B). It is interesting to note that GST-Rb was not phosphorylated as efficiently as most of the GST-BRCA1 constructs (Figure 4, lane 1).



B) **Average Phosphorylation**

GST-BRCA1 (1-500)	++++
GST-BRCA1 (452-1079)	++
GST-BRCA1 (1021-1552)	+
GST-BRCA1 (1501-1861)	+
GST-BRCA1 (504-802)	++
GST-BRCA1 (697-1276)	+

C) **Average Binding**

GST-BRCA1 (1-500)	+++
GST-BRCA1 (452-1079)	++
GST-BRCA1 (1021-1552)	+/-
GST-BRCA1 (1501-1861)	+
GST-BRCA1 (504-802)	++
GST-BRCA1 (697-1276)	+/-

Figure 4. Cyclin D1/cdk4 phosphorylates BRCA1 *in vitro*. A) Representative kinas assay. MCF-7 cell extracts (1 mg) were Immunoprecipitated with anti-cyclin D1 rabbit polyclonal antibodies overnight at 4°C. The complexes were precipitated with protein A+G agarose beads and washed twice with TNE300 + 0.1% NP-40, once with TNE50 + 0.1% NP-40, and twice with kinase buffer. Immune complexes were used for *in vitro* kinase assays using GST-Rb as a positive control, and various GST-BRCA1 fragments as substrates. Kinase reactions were separated on a 4-20 % Tris-glycine gel, dried, and exposed to a PhosphorImager cassette. B) Summary of Cyclin D1 phosphorylating various BRCA1 fragments. These results are representative of 4 independent experiments. C) Summary of Cyclin D1 and BRCA1 binding as demonstrated by the binding assays shown in Figure 2. Relative binding was calculated using ImageQuant Software. For relative BRCA1 binding: +++ = 7,000 ++ = 3,000, + =2,000 , and +/- =750 PhosphorImager units.

The average binding results are displayed in Figure 4C. The kinase assay results and *in vitro* binding results correlate nicely, in that the same regions important for binding are also highly phosphorylated. Again, it is the N-terminus (1-500) and the region of (504-802) of BRCA1 that appear to be important for the functional interaction between cyclin D1 and BRCA1. *In summary, the biochemical consequence of cyclin D1 interaction with BRCA1 is the phosphorylation of BRCA1, in particular the N-terminus aa 1-500.*

Task 2. The major goal of this task is to determine which cdk partner of cyclin D1 is responsible for phosphorylating BRCA1. In cell lines that overexpress cyclin D2, cyclin D2 has been shown to not only associate with cdk4 and cdk6, but also with cdk2 (14). In addition cdk2 complexes have been the major cyclin/cdk complexes shown to phosphorylated BRCA1 (34, 25). For these reasons it is important to determine which cdk is bound to the cyclin D1/BRCA1 complex in breast cancer cells.

Initially I chose to determine the cdk partner(s) of cyclin D1 in breast cancer cells. Asynchronous MCF-7 and T47D cells were harvest and processed for immunoprecipitation followed by western blot analysis. I observed, cyclin D1 in complex with cdk4, but not cdk6 (data not shown). Interestingly, a small subset of cyclin D1 could also be found in complex with cdk2 (data not shown), indicating that cyclin D1 can bind to both cdk4 and cdk2 in breast cancer cells.

Experiments were next performed to analysis if either cdk4 or cdk2 could be detected in complex with BRCA1. Asynchronous cells were immunoprecipitated with anti-BRCA1 antibodies and western blotted for both cdk4 and cdk2. This experiment revealed that both cdk4 and cdk2 could be detected in complex with BRCA1, but both at low levels (data not shown).

To differentiate between cdk2 and cdk4 kinase activity *in vivo*, a cdk4 specific drug, Fascaplysin (39) was obtained. Fascaplysin is a red pigment isolated from the marine sponge *Fascaplysinopsis sp.* It has been demonstrated to have an IC_{50} of 0.35 μM against cyclin D1/cdk4, but a much higher IC_{50} of greater than 50 μM against cdk2 complexes. Furthermore, Fascaplysin can inhibit cdk4 specific phosphorylation sites on the Rb *in vivo*. Therefore, Fascaplysin was utilized to verify that cyclin D1/cdk4 complexes phosphorylated BRCA1 *in vivo*. MCF-7 cells were treated for 24 hours with various concentrations of Fascaplysin (0.50, 0.75, 1.00, and 1.25 μM). Significant inhibition of BRCA1 phosphorylation was observed beginning with 0.75 μM of Fascaplysin (Figure 5A, lane 3). At 1.0 μM, increased inhibition was observed, but at 1.25 μM overall BRCA1 levels began to decrease possibly due to toxicity at this point. Phosphorylation at Ser-795 on Rb was used as a positive control for cdk4 inhibition. As can be seen in Figure 6B, Ser-795 phosphorylation was decreased at both 0.50 and 0.75 μM of Fascaplysin. Unexpectedly, Ser-795 phosphorylation dramatically increased at 1.00 μM Fascaplysin, but decreased at 1.25 μM (Figure 5B). Therefore, for further experiments, the optimal concentration of Fascaplysin to use will be 0.75 μM. *Collectively, these results indicate that cdk4 is the cyclin D1 partner responsible for BRCA1 phosphorylation and that this phosphorylation occurs in vivo.*

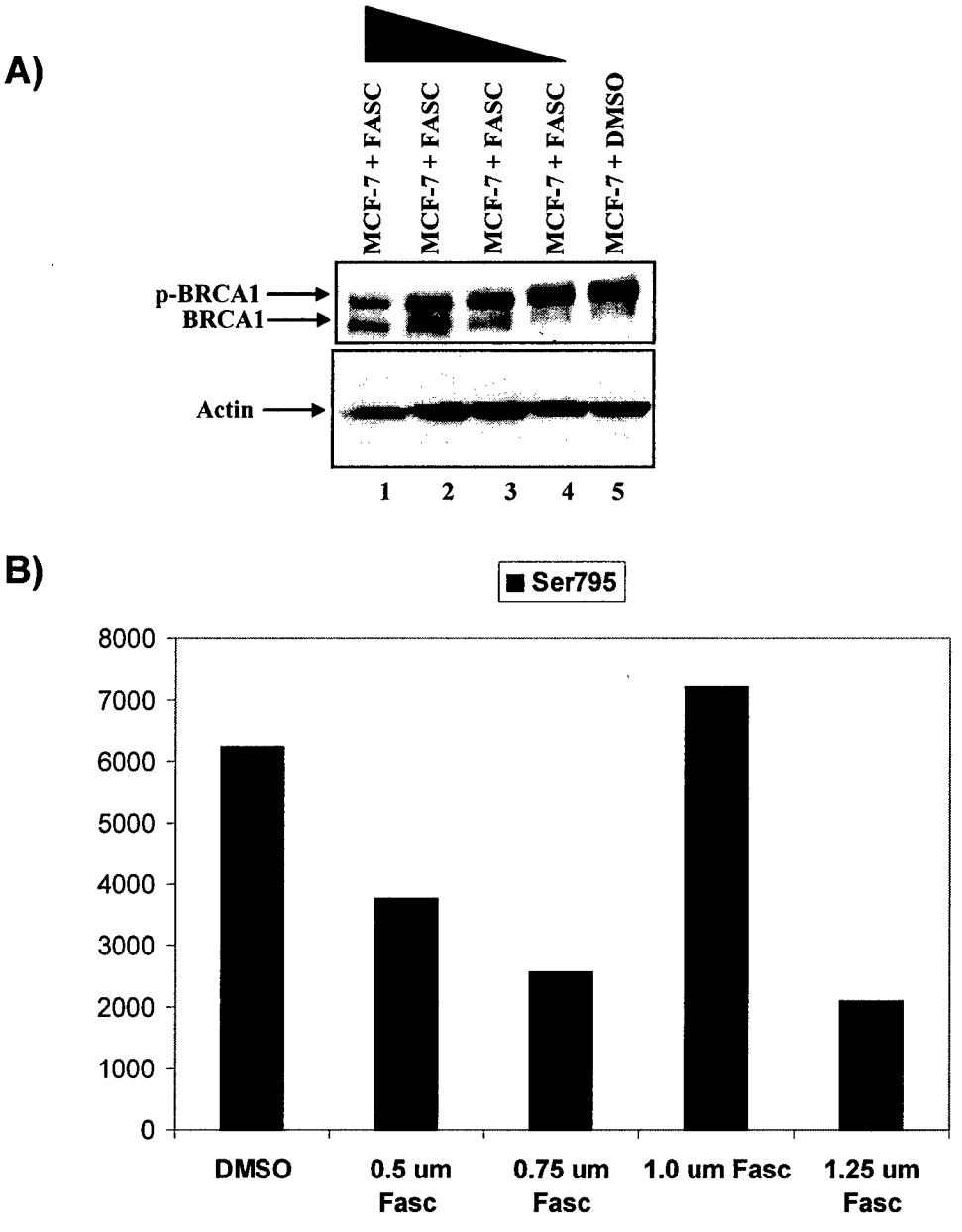


Figure 5: Cyclin D1/cdk4 phosphorylates BRCA1 *in vivo*. MCF-7 cells were treated with DMSO or varying concentrations of Fascalysin (0.5, 0.75, 1.00, and 1.25 micromole) for 24 hours and processed for Western Blotting. A) Western Blot analylsis using anti-BRCA1 rabbit polyclonal antibody after Fascaplysin treatment. B) Results of Western Blot analysis of phosphorylated Rb-Ser795 after Fascaplysin treatment.

Task 3: The purpose of this task is to determine which site(s) are critical for cyclin D1/cdk4 binding/phosphorylation of BRCA1. Originally, it was proposed that a series of BRCA1 cDNA constructs mutated within the RXL motifs would be constructed and then used for *in vitro* binding and *in vitro* kinase assays. The methodology of this task has been modified in that instead of cDNA constructs, peptides spanning the potential sites of binding/phosphorylation are being constructed. This new methodology is a more feasible alternative to provide us with valuable information on the specific sites critical for binding/phosphorylation of BRCA1. The peptides to be utilized are shown in Figure 6. These peptides were picked based on a number of factors. First, the regions BRCA1 (1-500) and (504-802) were shown to be important for both binding to cyclin D1 and being phosphorylated by cyclin D1/cdk4 (Figures 2 and 4). Secondly, cyclin/cdk complexes are known to phosphorylate their substrates through the use of a consensus cdk motif, Ser/Thr followed by Proline (indicated by Blue in Figure 6). This cdk consensus motif is often times preluded by a cyclin binding motif, RXL (indicated by Red in Figure 6) that is important for substrate docking and stability (40). Furthermore, the distance between the cdk consensus motif and the cyclin binding motif needs to be at least 12 amino acids apart in order to allow optimal phosphorylation to occur (41). It has been suggested that the RXL motif helps to specify which cdk phosphorylation site is targeted (41). In regions 1-500 and 504-802 of BRCA1, there are two spans of amino acids that fit the above criteria. We have obtained peptides spanning these regions (as indicated at the bottom of Figure 6). Mutated peptides where the Serine phosphorylation site is change to Alanine will be included as a negative control. These peptides will be used for *in vitro* kinase assays to verify that these regions are essential for cyclin D1/cdk phosphorylation of BRCA1. I foresee that I will have these results from these experiments by December 2005 (see Key Accomplishments, Figure 8).

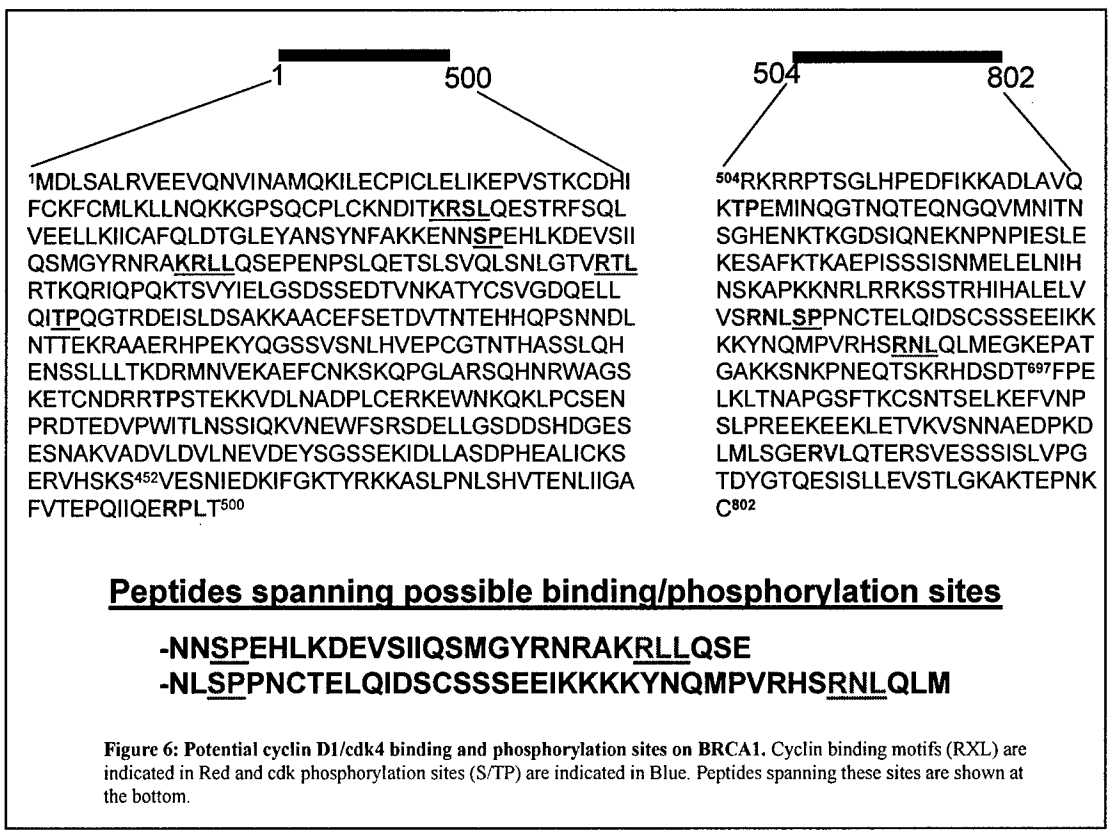


Figure 6: Potential cyclin D1/cdk4 binding and phosphorylation sites on BRCA1. Cyclin binding motifs (RXL) are indicated in Red and cdk phosphorylation sites (S/TP) are indicated in Blue. Peptides spanning these sites are shown at the bottom.

Aim 3. Determine the functional consequence of BRCA1 phosphorylation in breast cancer cells.

Task 1: At the time of my proposal, BRCA1 had been shown to bind preferentially to branched structures and long DNA stretches (42). Therefore, I had proposed to determine the effect of phosphorylation of BRCA1 by cyclin D1 complexes through the utilization of electromobility shift assays (EMSA) with multiple branched DNA structures. Since that time, BRCA1 has also been shown to bind to a specific DNA sequence, TTC(G/T)GTTG (43). Furthermore, the chromatin immunoprecipitation (ChIP) assay has become a widely used method to examine protein/DNA interaction *in vivo*. Thus, the methodology of this task has been changed in order to provide more valuable *in vivo* information about BRCA1 binding to specific DNA sequences.

Experiments have been initiated to examine the effects of cyclin D1/cdk4 phosphorylation on BRCA1 DNA binding. To this end, the cdk4 specific inhibitor, Fascaplysin was utilized. MCF-7 cells treated with Fascaplysin will have no or reduced cdk4 dependent BRCA1 phosphorylation, whereas control cells treated with DMSO will retain cdk4 specific BRCA1 phosphorylation. After treatment, cells were processed for ChIP. The antibodies targeted against cyclin D1, cdk4, and BRCA1 were used. In addition, a mouse monoclonal antibody (Tab 169) recognizing the viral protein Tax was used as a negative control. The primers were designed based on the Cable *et al.* paper in which they identified BRCA1 DNA binding consensus sites throughout the genome (43). pS2 primers were utilized as a positive control, because BRCA1 has been shown through ChIP to bind to this particular promoter (44). Unfortunately, after initial experiments, I have been unable to observe the presence of BRCA1 on the positive control nor in any of the experimental promoters, although I did observe cyclin D1 binding to some of the experimental promoters (date not shown). Thus, I am currently troubleshooting to find the cause of this unexpected result. A new BRCA1 antibody is being tested as antibody is extremely important in the ChIP procedure. I expect that these experiments will be completed by October of 2005 (see Key Accomplishments, Figure 8).

Task 2: Phosphorylation of BRCA1 has been shown to be cell cycle-dependent and induced by DNA damage (31, 45). Therefore, phosphorylation of BRCA1 may result in the alteration of the activity, as well as the subcellular localization of BRCA1. In particular, the phosphorylation of T508 by c-Akt results in the cytoplasmic accumulation of BRCA1 (46), whereas reports of BRCA1 phosphorylation induced by DNA damage have demonstrated mainly nuclear staining (47, 48). I have proposed to examine the consequence of cyclin D1/cdk phosphorylation of BRCA1 with regards to its subcellular location during progression through the cell cycle.

To begin this task, the localization of BRCA1 was examined in MCF-7 cells. Cells were fluorescently stained for cyclin D1, BRCA1, and the nucleus (Figure 7A, B, and C respectively). When red and green channels are merged (Figure 7D) or when all three channels are merged (Figure 7E), co-localization can be observed. Examples of co-localization have been indicated in Figure 7 by arrows. Co-localization was observed in the cytoplasm, in nuclear specals and in regions surrounding the nucleus.

Subsequent experiments looked at co-localization in both MCF-7 and MCF10A cells. Results of this experiment are shown in (Table 1). Co-localization could be observed in both cell types with a higher percentage being observed in MCF10A cells than MCF-7 cells (43% versus 29%). It was noted that cellular localization of BRCA1 is different between these two cell types.

MCF10A cells exhibited a more punctuate BRCA1 staining, whereas MCF-7 had a more diffuse mainly cytoplasmic staining pattern (Table 1). Because, MCF-10A cells express a lower level of cyclin D1 and thus lower activity of cyclin D1/cdk4 complexes, this may contribute to the difference between BRCA1 localization. Therefore, in subsequent experiments, MCF-7 cells will be treated with Fascaplysin to determine if inhibition of cyclin D1/cdk4 kinase activity results in differential BRCA1 cellular localization. *In summary thus far for this task, I have observed cyclin D1 and BRCA1 co-localization in both MCF-7 and MCF-10A cells.*

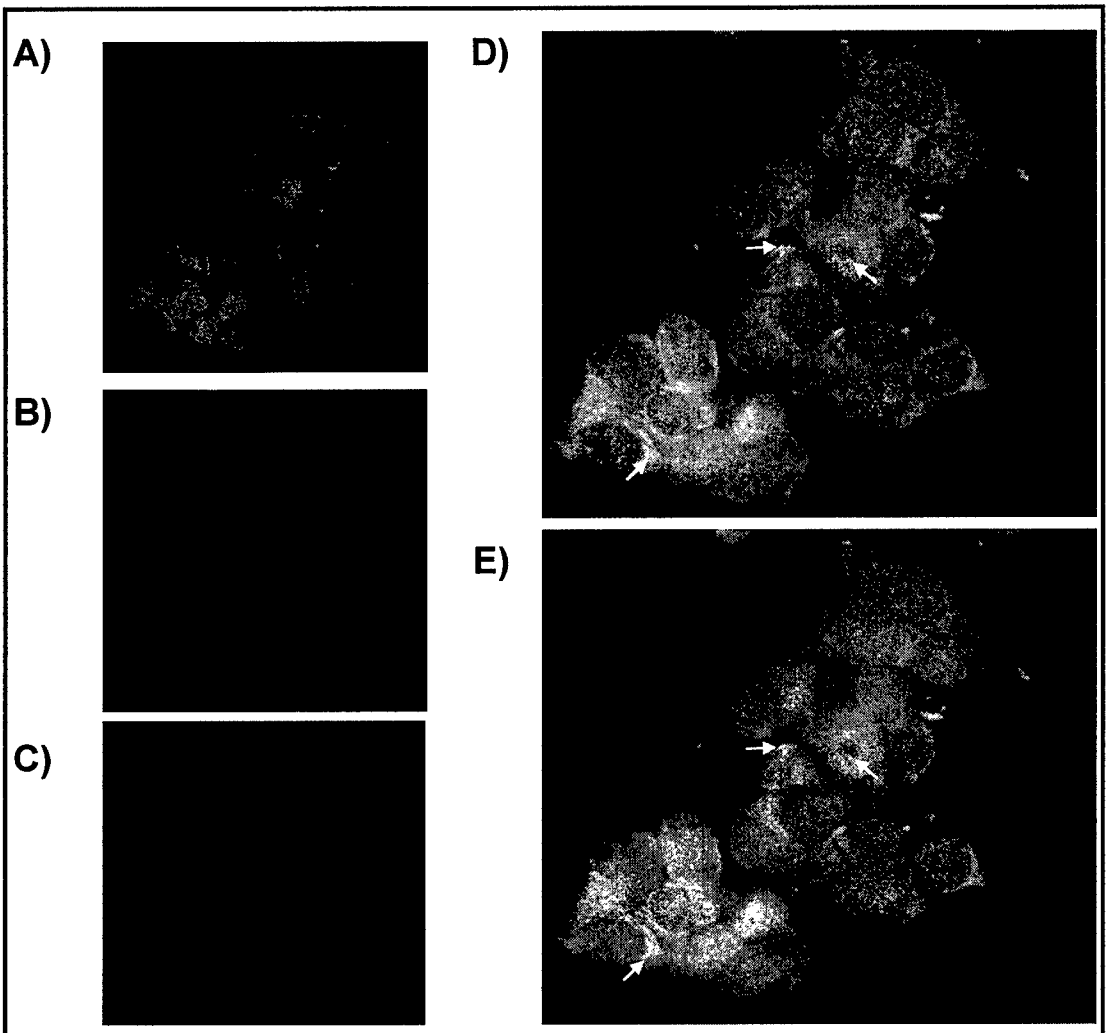


Figure 7: Cyclin D1 and BRCA1 co-localize in MCF-7 cells. Confocal optical sections (z=1.0 μm) are shown in all panels. A) Cyclin D1 staining as detected by rabbit polyclonal anti-cyclin D1 primary antibody and Alexa Fluor 488 secondary antibody. B) BRCA1 staining as detected by mouse monoclonal anti-BRCA1 primary antibody and Alexa Fluor 568 secondary antibody. C) Nuclear staining utlizing TOTO-3, a dimeric cyanine nucleic acid stain. D) Merged images of panels A and B. Arrows indicate points where colocalization is occurring, shown as yellow coloring when the two images are merged. E) Merged images of panels A, B, and C. Arrows indicate points where colocalization is occurring, shown as yellow coloring when the two images are merged.

TABLE 1: Cyclin D1 and BRCA1 Co-localize in MCF-7 and MCF-10A Cells

Cell Type	Total # of Cells	CycD1 location	BRCA1 location	# of Cells with Co-localization	% Co-localization
MCF-7	107	All N^1/C^2	Mainly C with little diffuse staining in N	32	Average=29%
MCFl0A	115	All N/C	N/C with punctuate N staining	50	Average =43%

[1]N=Nuclear [2]C=Cytoplasmic

KEY RESEARCH ACCOMPLISHMENTS

Displayed in Figure 8 is a timeline indicating which Aims and Tasks have been completed to date and which Task are still in progress.

1. Aim 1, Task 1 has been completed. I confirmed that BRCA1 and cyclin D1 interact in MCF-7 and T47D cells and showed that regions 1-500 and 504-802 of BRCA1 are particularly important for binding to cyclin D1 *in vitro*.
2. Aim 1, Task 2 has been completed. I have demonstrated that cyclin D1 and BRCA1 interact throughout all the stages of the cell cycle.
3. Aim 2, Task 1 has been completed. I have determined that the biochemical consequence of cyclin D1 interaction with BRCA1 is the phosphorylation of BRCA1, in particular the N-terminus (1-500).
4. Aim 2, Task 2 has been completed. I have established that cdk4 is the cyclin D1 partner responsible for BRCA1 phosphorylation and that this phosphorylation occurs *in vivo*.
5. Aim 2, Task 3 is in progress. I have narrowed down the specific sites that are important for cyclin D1/BRCA1 interaction/phosphorylation and I am currently confirming this information through experimental methods. This Task is expected to be completed by December, 2005.
6. Aim 3, Task 1 has been initiated. I have begun ChIP assays to determine is cyclin D1/cdk4 inhibition results in differential BRCA1 location on particular promoters. This Task is expected to be completed by October, 2005.
7. Aim 3, Task 2 is in progress. I have demonstrated that cyclin D1 and BRCA1 co-localized in both MCF-7 and MCF-10A cells. This Task is expected to be completed by June, 2005.

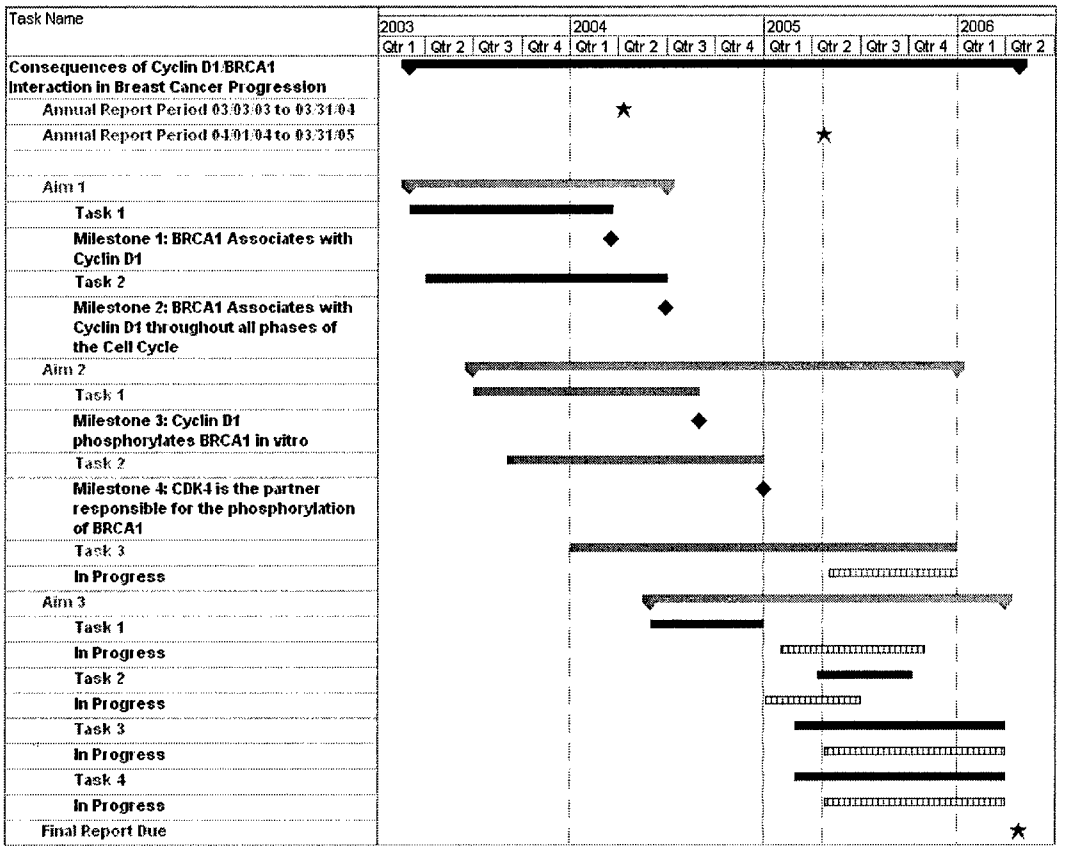


Figure 8: Timeline of progression with proposed Aims. Black bars indicate the time length of the DOD grant. Red stars indicate the points at which the annual report is due. Grey bars indicate the time length of the proposed Aims. Blue bars indicate the time length of the Tasks within Aim 1. Pink bars indicate the time length of the Tasks within Aim 2. Green bars indicate the time length of the Tasks within Aim 3. Black diamonds indicate the milestone achievements, where the Task has been completed. Hatched bars, both pink and green, indicate that the task is currently in progress, and the time expected to complete the proposed Task.

REPORTABLE OUTCOMES

During the report time frame of March 2004-2005 the following reportable outcomes have been made:

1. An abstract was submitted for George Washington Medical Center's 10th Annual Research Day 2005 based on this work. I was awarded the opportunity to present an Oral Presentation, in which I subsequently was awarded first place for the talk.

2. An abstract was submitted for the DOD Era of Hope 2005 Conference.

3. Currently, there is a manuscript in preparation based on this work that should be completed by August, 2005.

4. I have been awarded the Sallie Rosen Kaplan Fellowship for woman interested in pursuing careers in cancer research. The award is from NIH/NCI for 1-2 top scientists in the nation interested in performing cancer research for 3-5 year at the NIH Bethesda Campus. I won the award from a pool of 50 applicants for the year 2005 fiscal year. I have since declined the award, but the DOD predoctoral fellowship was a key factor in my being awarded this prestigious fellowship.

5. I have obtained a postdoctoral position at Virginia Commonwealth University in Dr. Steven Grant's laboratory. Again, the DOD predoctoral fellowship has been instrumental in me obtaining this position.

CONCLUSIONS

My second annual DOD report has indicated multiple findings and conclusions. First, I have confirmed that BRCA1 and cyclin D1 interact in MCF-7, T47D, and MCF-10A cells. This complex is highly stable and was detected in asynchronous cell populations. This *in vivo* interaction could also be detected through confocal microscopy showing co-localization of cyclin D1 and BRCA1 in both MCF-7 and MCF-10A cells. *In vitro* binding assays utilizing ^{35}S-cyclin D1 showed that regions 1-500 and 504-802 of BRCA1 are particularly important for binding to cyclin D1 *in vitro*. Second, I have demonstrated that cyclin D1 and BRCA1 interact throughout all the stages of the cell cycle. This observation suggests that the interaction between cyclin D1 and BRCA1 is either important throughout the cell cycle, or that other circumstances, such as phosphorylation or protein binding partners, render this interaction functional.

Along these same lines, I have determined that the biochemical consequence of cyclin D1 interaction with BRCA1 is the phosphorylation of BRCA1. *In vitro* kinase assays indicated that the N-terminus (1-500) of BRCA1 was consistently highly phosphorylated. In addition, the region 452-1079 and the smaller region contained within, 504-802, exhibited high levels of phosphorylation. This data suggests that there is more than one site of phosphorylation for cyclin D1/cdk complexes. It is possible that one site of phosphorylation may be more important than the other depending on the stages of the cell cycle. The specific sites that are important for cyclin D1/BRCA1 interaction have also been identified using bioinformatics methods. I am currently confirming this information through experimental methods, such as *in vitro* kinase assays with peptides spanning the regions of interest.

I also established that cdk4 is the cyclin D1 partner responsible for BRCA1 phosphorylation. Cyclin D1/cdk4 complexes were observed phosphorylating BRCA1 both *in vitro* and *in vivo*. This is the first time to my knowledge that cdk4 has been shown to phosphorylate BRCA1 *in vivo*.

Finally, I have initiated two different approaches to determine the functional consequences of the phosphorylation of BRCA1 by cyclin D1/cdk4. ChIP assays are being performed to determine is cyclin D1/cdk4 inhibition results in differential BRCA1 location on particular promoters. Drug inhibition of cyclin D1/cdk4 complexes followed by fluorescent staining for BRCA1 localization has begun. These studies will indicate whether phosphorylation of BRCA1 alters its subcellular localization. The results of these studies will be presented in my third and final DOD report and should complete my proposed tasks.

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